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Supporting Information

Bicyclic Imidazolium Inhibitors of Gli Transcription Factor Activity

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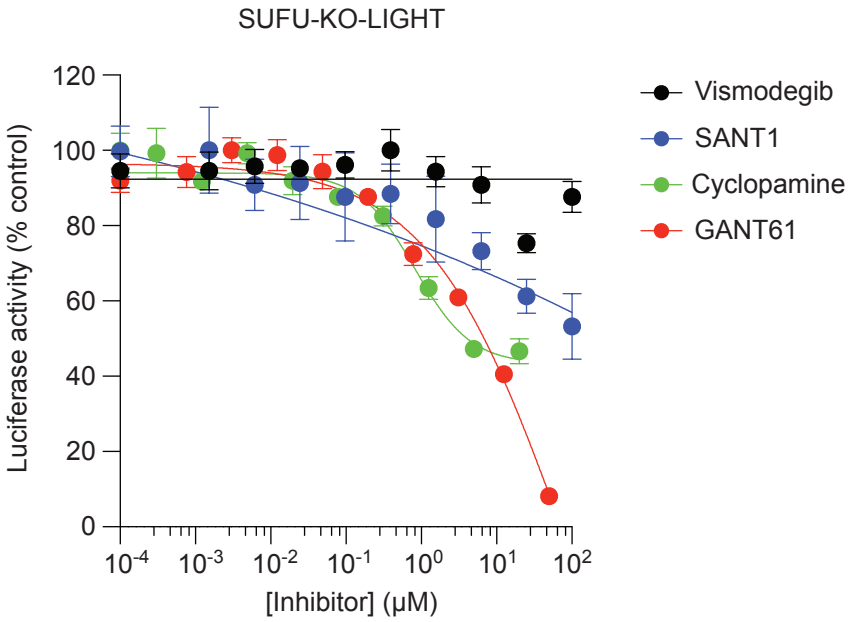


Figure S1. SMO inhibitors do not inhibit Gli-dependent luciferase activity in SUFU-KO-LIGHT cells. Cells were incubated with the indicated inhibitors for 24 h, and the resulting luciferase reporter activities were measured. Data are the average of three biological replicates ± s.e.m.

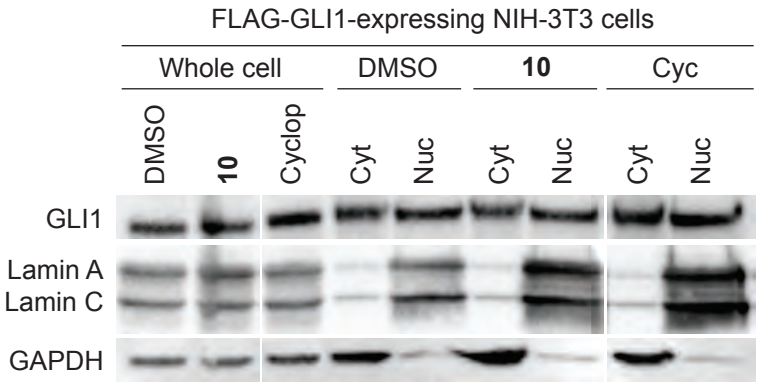


Figure S2. Bicyclic imidazoliums do not alter the subcellular localization of FLAG-GLI1. Western blot analyses of FLAG-GLI1-expressing NIH-3T3 cells treated with either 5 μM **10**, 5 μM cyclopamine (Cyc), or DMSO vehicle for 4 h and then lysed and fractionated by centrifugation. Subcellular fractionation was confirmed using lamins and GAPDH as nuclear and cytosolic markers, respectively. A representative blot from three biological replicates is shown, and all samples were analyzed on the same blot.

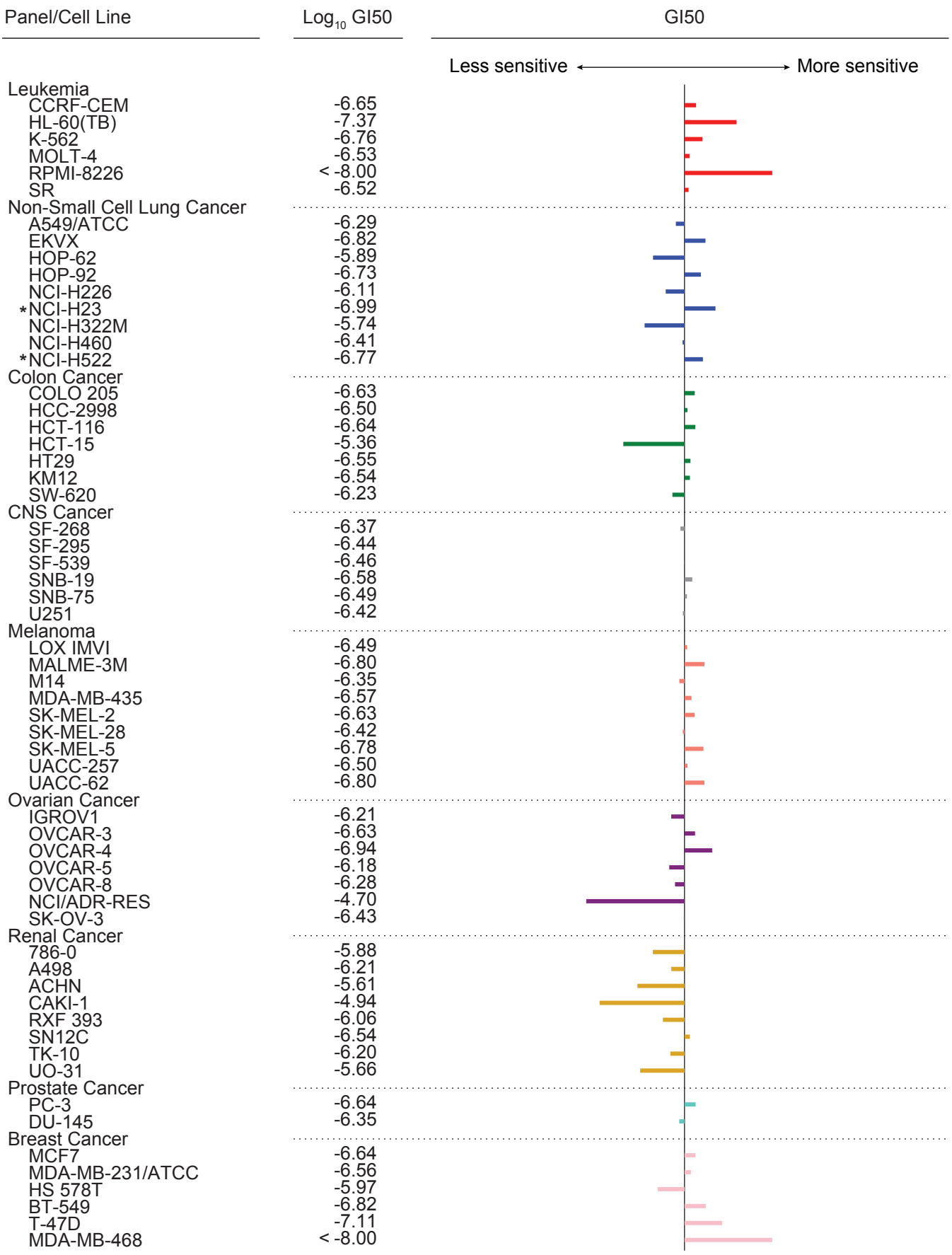


Figure S3. Activity profile of imidazolium 5 against the NCI60 cancer cell lines. Compound concentrations required for 50% of maximal inhibition (GI50s) are shown. The H23 and H522 lines (asterisks) express high levels of *GLI1.*

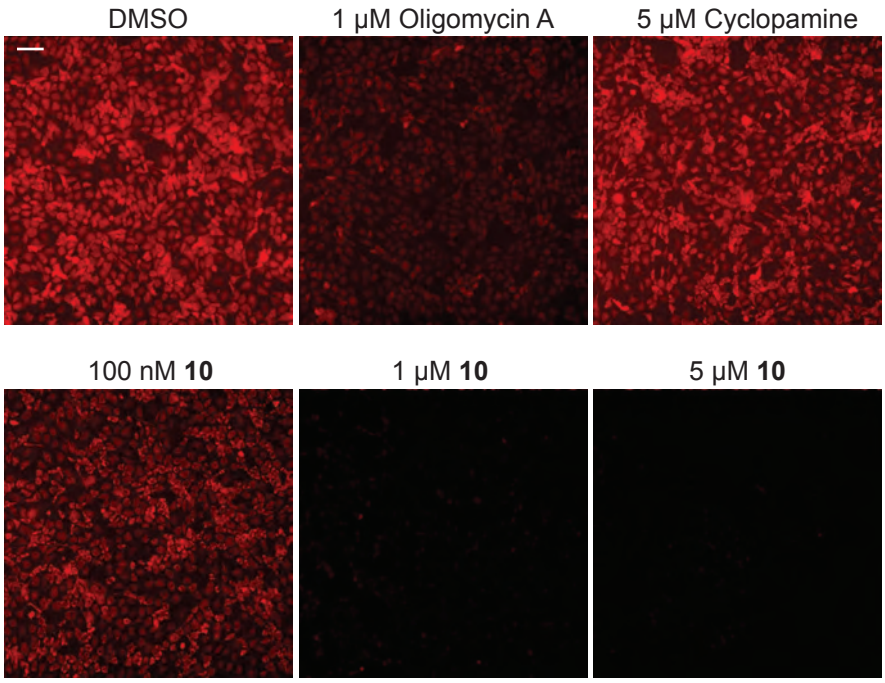


Figure S4. Bicyclic imidazoliums can disrupt mitochondrial membrane potential. NIH-3T3 cells were treated with the designated compounds for 24 h and then stained for 30 min with 500 nM tetramethylrhodamine (TMRM), which selectively labels mitochondria with a membrane potential. Scale bar: 100 μm.

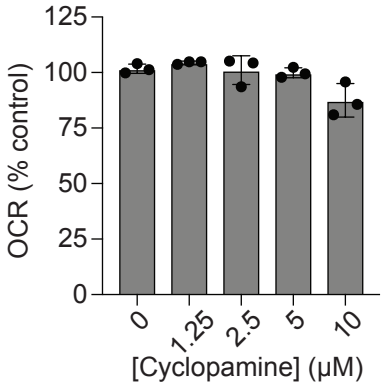
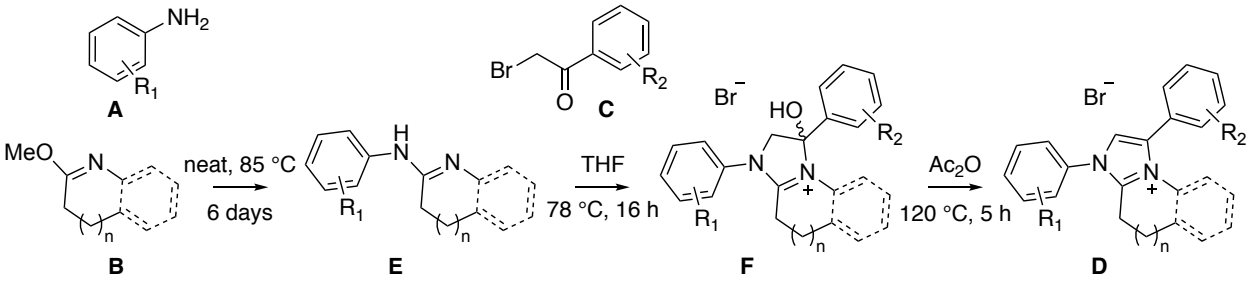


Figure S5. Hh pathway inhibition does not alter mitochondrial respiration. Oxygen consumption rates (OCRs) of NIH-3T3 cells treated with varying doses of cyclopamine, as measured by the Seahorse Mito Stress assay. The SMO antagonist did not significantly inhibit OXPHOS as determined by one-way ANOVA against DMSO. Data are the average of three biological replicates ± s.e.m.

General Methods. All reactions were performed in thick-walled pressure vessels under a positive pressure of nitrogen. Stainless steel syringes or cannulae were used to transfer air- and moisture sensitive liquids. Organic solutions were concentrated on Büchi R-200 rotary evaporators at ~20 Torr (house vacuum) at 25–35 °C, then at ~1 Torr (vacuum pump) unless otherwise indicated. Column chromatography was performed using 200−300 mesh silica gel. Commercial reagents and solvents were used as received unless otherwise noted. Compounds **1**, **4**, **11**, and **12** were commercially available; the synthesis of **2**, **3**, **6**, and **8** was contracted to Chao Che (Peking University); and the synthesis of **13** and **14** was contracted to Kemio Solutions (Bangalore, India). Compounds **5**, **7**, **9**, and **10** were synthesized in-house.

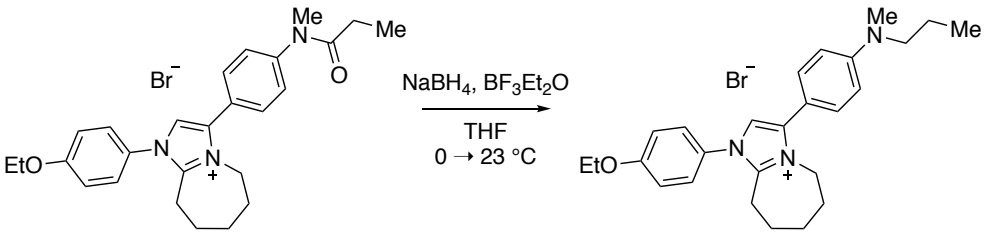
Instrumentation. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a Bruker 300, Bruker 500, or Varian 500 INOVA spectrometer. High resolution mass spectra (HRMS) were obtained on a quadrupole time-of-flight (QTOF) mass spectrometer. Chemical shifts are reported in parts per million from internal tetramethylsilane on the δ scale and are referenced from the residual protium in the NMR solvent ($CHCl_3$: δ 7.27 ppm, CD_2HOD: δ3.31 ppm, CD_2HCN: δ1.94 ppm).

General Procedure for the Synthesis of Bicyclic Imidazolium Compounds (D):



A mixture of aniline **A** (1.0 equiv) and azepine **B** (1.0 equiv) was heated to 85 ºC and stirred under a nitrogen atmosphere for 6 days. The resulting precipitate was collected by vacuum filtration and washed with 3×3 mL Et_2O to yield a 7-amino-azepine intermediate **E**. 2-Bromoketone **C** (1.0 equiv) was added to a pressure vessel charged with a suspension of the corresponding 7-amino-azepine intermediate **E** (1.0 equiv) in THF (to 2.0 M) and the vessel was sealed and placed on an oil bath at 78 °C. The reaction mixture was stirred at 78 °C for 16 h, then allowed to cool to room temperature and the solution was concentrated under reduced pressure. The crude hydrate **F** was dissolved in acetic anhydride (to 1.5 M) in a pressure vessel, and the vessel was sealed and maintained on an oil bath at 120 °C for 5 h. The vessel was then allowed to cool to room temperature and the reaction mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (MeOH:CH_2Cl_2, 1:20) to give bicyclic imidazolium compound **D**.

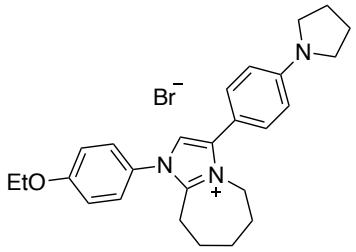
Characterization data



S1 **2**

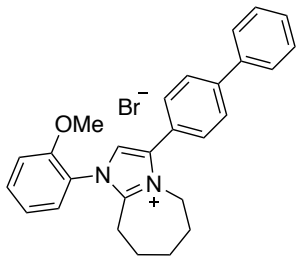
Bicyclic imidazolium compound **S1** was prepared according to the General Procedure with **A** = 4-ethoxyaniline, **B** = 7-methoxy-3,4,5,6-tetrahydro-2*H*-azepine, and **C** = *N*-(4-(2-bromoacetyl)phenyl)-*N*-methylpropionamide, and used directly in the next step. To the solution of **(S1)** (30 mg) in 5 mL of anhydrous THF was added $NaBH_4$ (40 mg) and BF_3OEt_2 (0.2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight. The reaction was then quenched with saturated aqueous $NaHCO_3$ (5 mL), and extracted with CH_2Cl_2 (3×10

mL). The combined organic phase was concentrated under reduced pressure, and the residue was purified by flash column chromatography on silica gel (MeOH:CH_2Cl_2, 1:20) to yield desired product **2**. Yield = 87%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (*d, J* = 8.8 Hz, 2H), 7.30 (*d, J* = 8.8 Hz, 2H), 7.04 (*d, J* = 8.8 Hz, 2H), 6.95 (*s,* 1H), 6.74 (*d, J* = 8.8 Hz, 2H), 4.40 (*t*, *J* = 4.4 Hz, 2H), 4.12 (*q*, *J* = 6.8 Hz, 2H), 3.35 (*t*, *J* = 7.2 Hz, 2H), 3.26 (*t*, *J* = 5.2 Hz, 2H), 3.00 (*s*, 3H), 2.03 (*d*, *J* = 4.0 Hz, 2H), 1.84 (*m*, 2H), 1.74 (*m*, 2H), 1.59-1.68 (*m,* 2H), 1.47 (*q*, *J* = 6.8 Hz, 3H), 0.97 (*t*, *J* = 7.2 Hz, 3H); HRMS (*m/z*) calc. for $C_{26}H_{34}N_3O$ (+) 404.2696, found 404.2694.



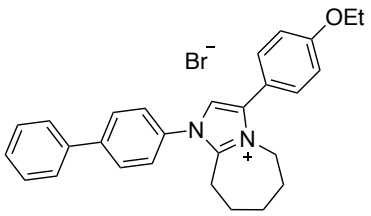
3

Bicyclic imidazolium compound **3** was prepared according to the General Procedure with **A** = 4-ethoxyaniline, **B** = 7-methoxy-3,4,5,6-tetrahydro-2*H*-azepine, and **C** = 2-bromo-1-(4-(pyrrolidin-1-yl)phenyl)ethan-1-one. Yield = 75%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50-7.55 (*m,* 2H), 7.26-7.30 (*m*, 2H), 7.00-7.06 (*m*, 3H), 6.63 (*d*, *J* = 8.7 Hz, 1H), 6.44 (*s*, 2H), 4.43-4.46 (*m*, 2H), 4.13 (*q*, *J* = 6.9 Hz, 2H), 3.31-3.35 (*m*, 4H), 1.76-2.08 (*m*, 12H), 1.47 (*t*, *J* = 6.9 Hz, 2H); HRMS (*m/z*) calc. for $C_{26}H_{32}N_3O$ (+) 402.2540, found 402.2535.



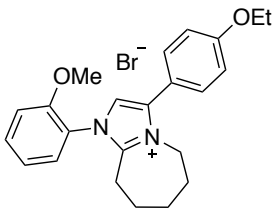
5

Bicyclic imidazolium compound **5** was prepared according to the General Procedure with **A** = 2-methoxyaniline, **B** = 7-methoxy-3,4,5,6-tetrahydro-2*H*-azepine, and **C** = 1-([1,1'-biphenyl]-4-yl)-2-bromoethan-1-one. Yield = 89%. ^{1}H NMR (500 MHz, $CDCl_3$) 7.75 (*d*, *J* = 8.0 Hz, 2H), 7.45-7.63 (*m*, 8H), 7.39 (*tt, J* = 7.4, 1.2 Hz, 1H), 7.11-7.16 (*m*, 2H), 7.11 (*s*, 1H), 4.22-4.40 (*m*, 2H), 3.88 (*s*, 3H), 2.90-3.04 (*m*, 2H), 1.64-2.10 (*m*, 6H); HRMS (*m/z*) calc. for $C_{27}H_{27}N_2O$ (+) 395.2118, found 395.2119.



6

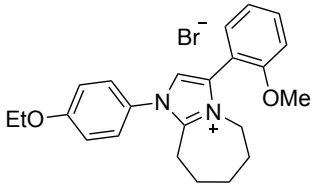
Bicyclic imidazolium compound **6** was prepared according to the General Procedure with **A** = [1,1'-biphenyl]-4-amine, **B** = 7-methoxy-3,4,5,6-tetrahydro-2*H*-azepine, and **C** = 2-bromo-1-(4-ethoxyphenyl)ethan-1-one. Yield = 55%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75-7.80 (*m*, 4H), 7.59-7.61 (*m*, 2H), 7.46-7.49 (*m*, 4H), 7.40-7.43 (*m*, 1H), 7.10 (*s*, 1H), 6.99 (*d*, *J* = 9.0 Hz, 2H), 4.46 (*t*, *J* = 5.0 Hz, 2H), 4.07 (*q*, *J* = 7.0 Hz, 2H), 3.46 (*t*, *J* = 5.5 Hz, 2H), 2.05-2.08 (*m*, 2H), 1.89-1.91 (*m*, 2H), 1.81-1.82 (*m*, 2H), 1.43 (*t*, *J* = 7.0 Hz, 3H); HRMS (*m/z*) calc. for $C_{28}H_{29}N_2O$ (+) 409.2274, found 409.2272.



7

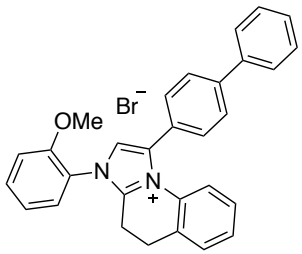
Bicyclic imidazolium compound **7** was prepared according to the General Procedure with **A** = 2-methoxyaniline, **B** = 7-methoxy-3,4,5,6-tetrahydro-2*H*-azepine, and **C** = 2-bromo-1-(4-

ethoxyphenyl)ethan-1-one. Yield = 98%. ^{1}H NMR (500 mHz, $CDCl_3$) ∂ 7.55 (*td*, *J* = 7.9, 1.5 Hz), 7.45 (*dd*, *J* = 8.2, 1.5 Hz, 1H), 7.34 (*d*, *J* = 8.6 Hz, 2H), 7.13 (*d*, *J* = 8.2 Hz, 1H), 7.10–7.14 (*m*, 1H), 7.00 (*d*, *J* = 8.6 Hz, 2H), 7.01 (*s*, 1H), 4.24–4.29 (*m*, 2H), 4.06 (*q*, *J* = 7.0 Hz, 2H), 3.86 (*s*, 3H), 2.87–3.02 (*m*, 2H), 1.62–2.09 (*m*, 6H), 1.41 (*t*, *J* = 7.0 Hz, 3H); HRMS (*m/z*) calc. for $C_{23}H_{27}N_2O_2$ (+) 363.2067, found 363.2067.



8

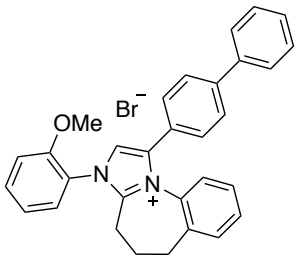
Bicyclic imidazolium compound **8** was prepared according to the General Procedure with **A** = 4-ethoxyaniline, **B** = 7-methoxy-3,4,5,6-tetrahydro-2*H*-azepine, and **C** = 2-bromo-1-(2-methoxyphenyl)ethan-1-one. Yield = 73%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (*d*, *J* = 9.0 Hz, 2H), 7.49-7.53 (*m*, 2H), 7.08 (*t*, *J* = 6.5 Hz, 1H), 7.00-7.06 (*m*, 4H), 4.23 (*t*, *J* = 5.0 Hz, 2H), 4.07 (*q*, *J* = 7.0 Hz, 2H), 3.88 (*s*, 3H), 3.40 (*t*, *J* = 5.0 Hz, 2H), 2.06-2.09 (*m*, 2H), 1.88-1.90 (*m*, 2H), 1.79-1.82 (*m*, 2H), 1.42 (*t*, *J* = 7.0 Hz, 3H); HRMS (*m/z*) calc. for $C_{23}H_{27}N_2O_2$ (+) 363.2067, found 363.2068.



9

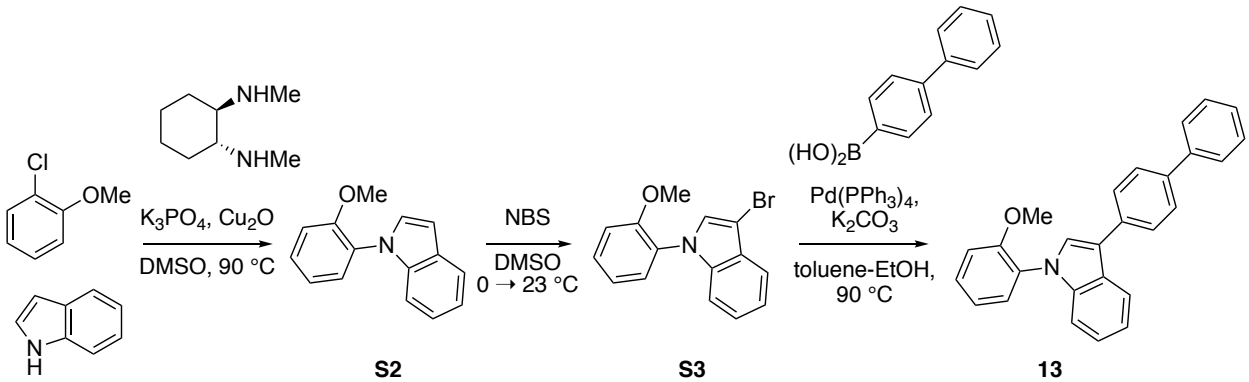
Bicyclic imidazolium compound **9** was prepared according to the General Procedure with **A** = 2-methoxyaniline, **B** = 2-methoxy-3,4-dihydroquinoline,[1] and **C** = 1-([1,1'-biphenyl]-4-yl)-2-bromoethan-1-one. Yield = 40%. ^{1}H NMR (500 MHz, CD_3CN) δ 7.90 (*m*, 2H), 7.76 (*m*, 4H), 7.66

(*m*, 2H), 7.58 (*m*, 4H), 7.50 (*m*, 1H), 7.37-7.44 (*m*, 2H), 7.29 (*m,* 1H), 7.22 (*m*, 1H), 7.03 (*d*, *J* = 8.5 Hz, 1H), 3.10-3.24 (*m*, 4H); HRMS (*m/z*) calc. for $C_{30}H_{25}N_2O$ (+) 429.1961, found 429.1958.



10

Bicyclic imidazolium compound **10** was prepared according to the General Procedure with **A** = 2-methoxyaniline, **B** = 2-methoxy-4,5-dihydro-3*H*-benzo[*b*]azepine,[2] and **C** = 1-([1,1'-biphenyl]-4-yl)-2-bromoethan-1-one. Yield = 74%. ^{1}H NMR (500 MHz, $CDCl_3$, rotamers) 7.98-8.19 (*m*, 0.5H), 7.23-7.68 (*m*, 15H), 7.20 (*d*, *J* = 8.6 Hz, 1H), 7.01-7.12 (*br s*, 1H), 6.49-6.69 (*br s*, 0.5 H), 3.97 (*s*, 3H), 2.27-3.09 (*m*, 6H). HRMS (*m/z*) calc. for $C_{31}H_{27}N_2O$ (+) 443.2118, found 443.2112.

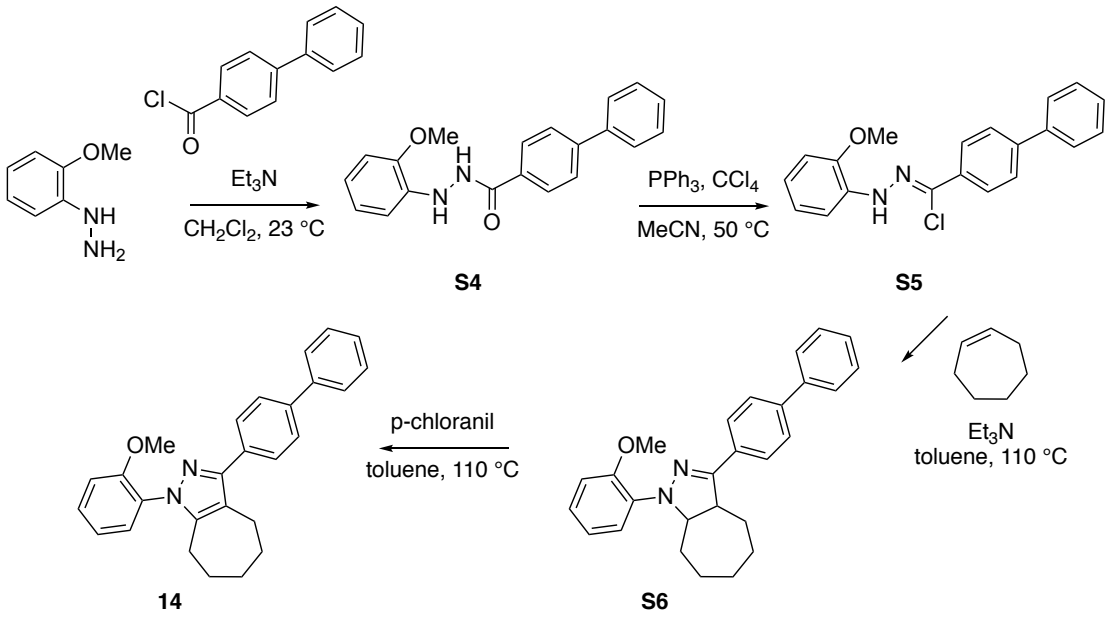


Indole **13**. To a solution of 1H-indole (2.0 g) in DMSO (20 mL) was added 1-chloro-2-methoxybenzene (2.92 g), K_3PO_4 (7.21 g), and copper oxide (0.424 g), followed by (1*R*,2*R*)-N1,N2-dimethylcyclohexane-1,2-diamine (0.24 g) at room temperature. The reaction mixture was

stirred at 90 °C for 16 h, then cooled to room temperature, quenched with water, and extracted with ethyl acetate. The combined extracts were washed with water, brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (EtOAc:hexanes, 3:7) to yield desired product **S2**. Yield = 34%.

To a solution of 1-(2-methoxyphenyl)-1H-indole (**S2**, 1.0 g) in DMF (10 mL) was added *N*-bromosuccinimide (1.19 g) at 0 °C and stirred at room temperature for 6 h. The reaction was quenched with water and extracted with CH_2Cl_2. The combined extracts were washed with water and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (EtOAc:hexanes, 1:9) to yield desired product **S3**. Yield = 64%.

To a solution of 3-bromo-1-(2-methoxyphenyl)-1H-indole (**S3**, 0.20 g) and [1,1'-biphenyl]-4-ylboronic acid (0.14 g) in 1:1 toluene:EtOH (4 mL) was added K_2CO_3 (0.22 g) and the mixture was purged with nitrogen for 5 minutes. $Pd(PPh_3)_4$ was added and the mixture was stirred at 90 °C for 4 h. The reaction was quenched with water and extracted with EtOAc. The combined extracts were washed with water, brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (EtOAc:hexanes, 3:7) to yield desired product **13**. Yield = 20%. ^{1}H NMR (400 MHz, $CDCl_3$) δ8.03 (dd, J = 6.4, 2.8 Hz, 1H), 7.81 (d, J = 8.2 Hz, 3H), 7.68 (dd, J = 11.8, 7.9 Hz, 3H), 7.53 – 7.41 (m, 3H), 7.44 – 7.31 (m, 2H), 7.25 (ddd, J = 14.6, 6.1, 3.4 Hz, 2H), 7.16 – 7.06 (m, 1H), 3.81 (s, 2H), 1.35 (d, J = 15.3 Hz, 1H), 1.29 (s, 1H), 1.27 (d, J = 11.8 Hz, 2H), 0.91 – 0.82 (m, 1H). HRMS (*m/z*) calc. for $C_{27}H_{22}NO$ (+) 376.1618, found 376.1616.



Pyrazole **14**. To a solution of 4-(pyridin-3-yl)benzoyl chloride (0.80 g) in CH_2Cl_2 (8.0 mL), (2-methoxyphenyl)hydrazine (0.50 g) was added, then Et_3N (0.72 g) was added dropwise. The reaction mixture was stirred at room temperature for 1.5 h, then an additional (2-methoxyphenyl)hydrazine (0.50 g) and Et_3N (0.72 g) were added and the mixture was stirred at room temperature for 16 h. The reaction was quenched with aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was concentrated to yield **S4**, which was used in the next step without further purification.

To a solution of **S4** (0.50 g) and triphenylphosphine (0.48 g) in acetonitrile (2 mL) was added CCl_4 (2 mL), and the reaction mixture was stirred at room temperature for 1 h and then at 50 °C for 16 h. The reaction was quenched with water and extracted with EtOAc. The combined extracts were washed with water, brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (EtOAc:hexanes, 3:7) to yield desired product **S5**. Yield = 68%.

To a solution of **S5** (0.35 g) in toluene (4 mL) was added cycloheptene (0.49 g), Et_3N (0.40 mL), and the mixture was stirred at 110 °C for 3 h, then at room temperature for 16 h. The reaction was quenched with water and extracted with EtOAc, the combined extracts were washed with water, brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (EtOAc:hexanes, 3:7) to yield desired product **S6**. Yield = 38%.

To a stirred mixture of **S6** (150 mg) in toluene (2 mL), was added *p*-chloranil (101 mg) and the mixture was heated to 110 °C for 3 h. The reaction was quenched with water and extracted with EtOAc. The combined extracts were washed with water, brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (EtOAc:hexanes, 1:4), followed by preparative thin-layer chromatography (EtOAc:hexanes, 3:20) to give the desired product **14**. Yield = 63%. ^{1}H NMR (400 MHz, $CDCl_3$) δ7.73 – 7.66 (m, 3H), 7.63 (dd, J = 8.1, 1.5 Hz, 6H), 7.47 – 7.42 (m, 4H), 7.42 – 7.36 (m, 2H), 7.36 – 7.28 (m, 2H), 7.09 – 6.98 (m, 3H), 3.81 (s, 4H), 2.83 (t, J = 5.6 Hz, 3H), 2.61 (s, 1H), 2.53 (s, 1H), 1.91 – 1.82 (m, 3H), 1.55 (s, 2H). HRMS (*m/z*) calc. for $C_{27}H_{28}N_2O$ (+) 396.2117, found 39.2118.

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[1] T. Tsuritani, Y. Yamamoto, M. Kawasaki, T. Mase, *Org. Lett.* **2009**, *11*, 1043–1045.
[2] D. R. Cheshire, *IDrugs*, **2001**, *4*, 795–803.

Current Data Parameters

EXPNO 1
PROCNO 1

F2 - Acquisition Parameters

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DE	6.50 usec
TE	298.7 K
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F2 - Processing parameters

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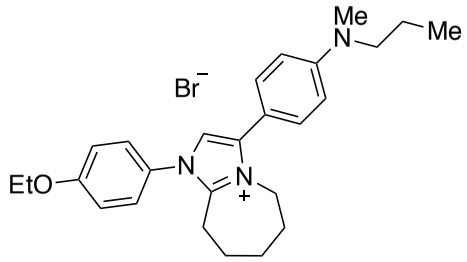
PC	1.00

7.493 7.471 7.299 7.277 7.268 7.040 7.018 6.953 6.737 6.715 4.404 4.393 4.382 4.115 4.098 4.080 4.063 3.346 3.328 3.309 3.264 3.251 3.238 2.996 2.030 2.020 1.837 1.739 1.682 1.663 1.644 1.626 1.607 1.589 1.469 1.452



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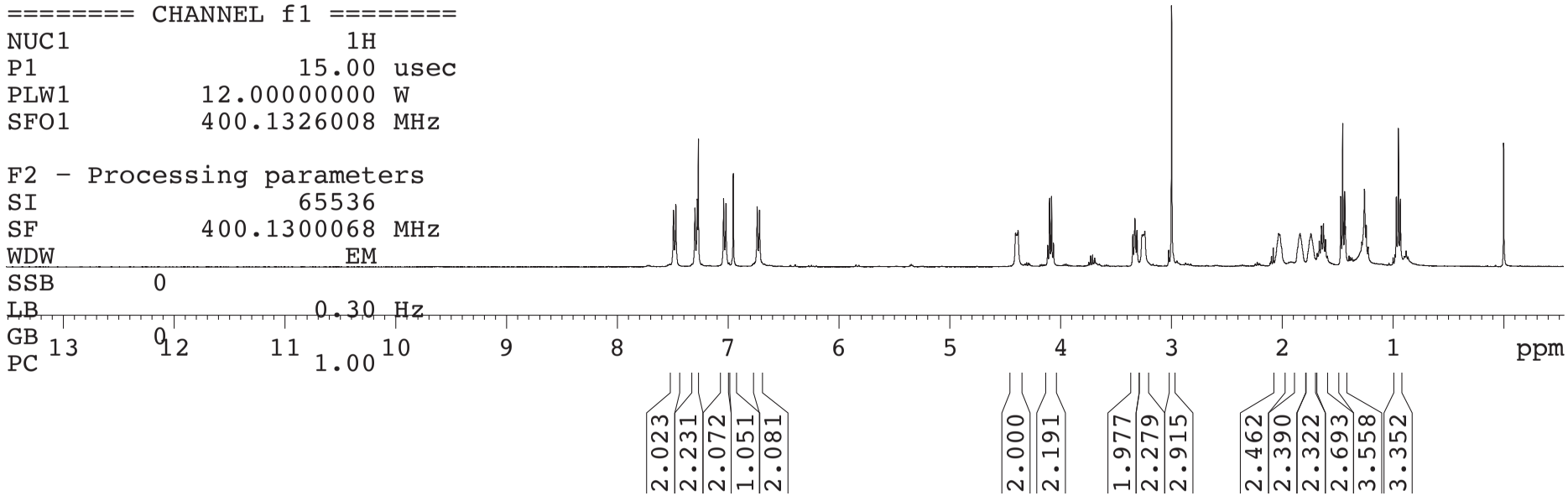

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RG	71.8
DW	83.400 usec
DE	6.50 usec
TE	294.2 K
D1	2.00000000 sec
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SFO1	300.1318458 MHz
SI	32768
SF	300.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

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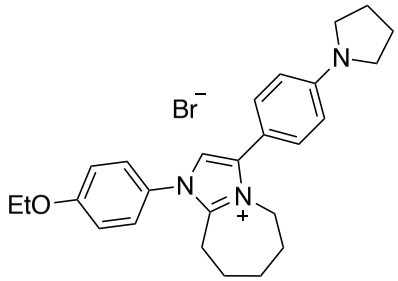

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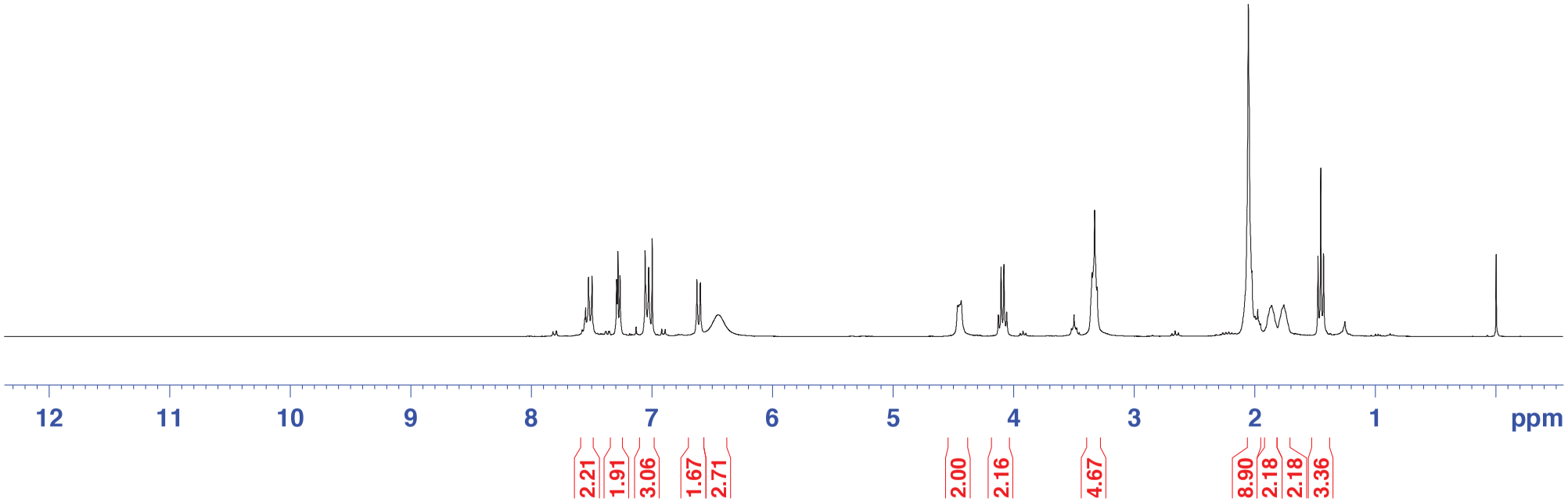

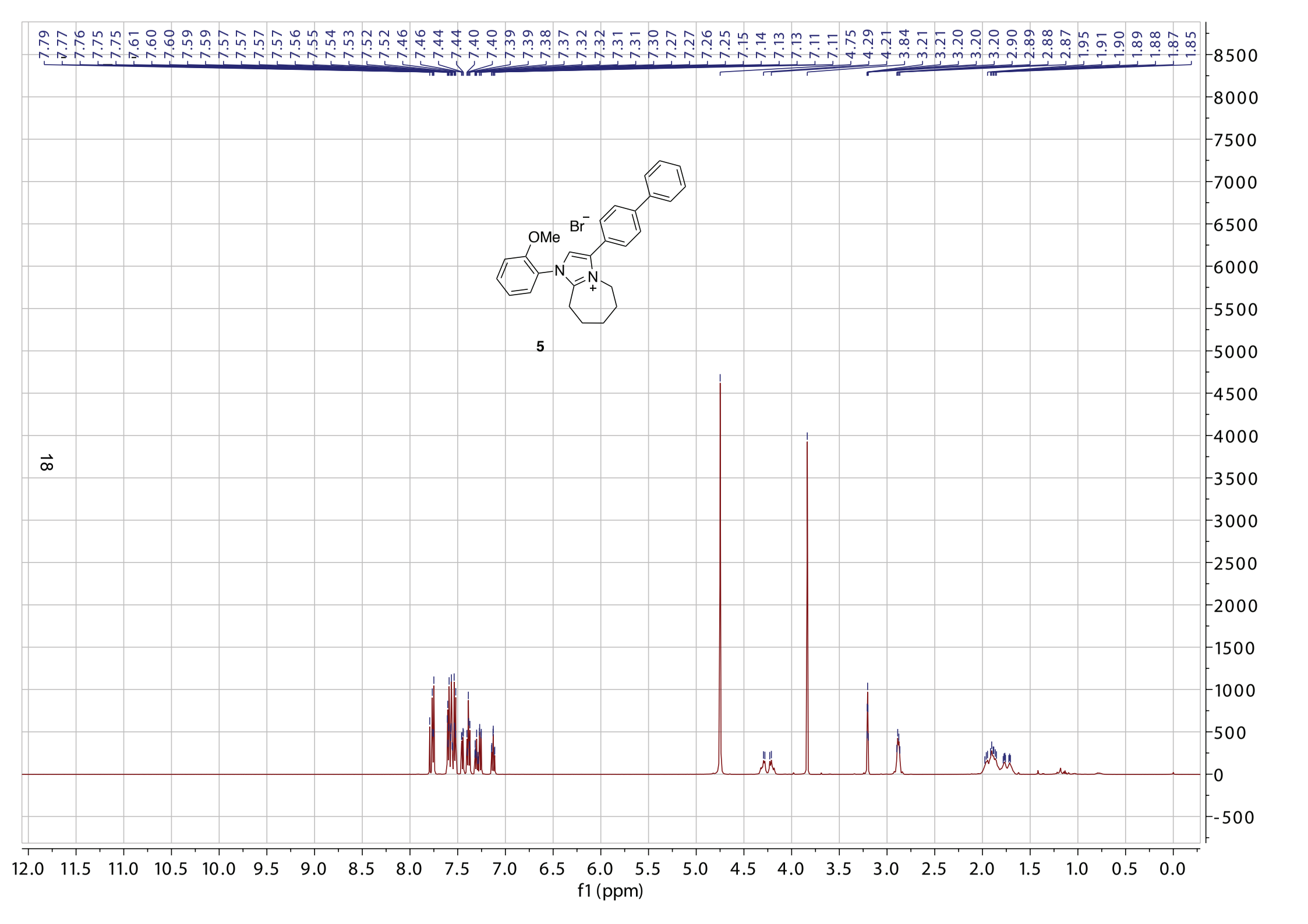
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OMe
Br⁻
N
N+
5
18
8500 8000 7500 7000 6500 6000 5500 5000 4500 4000 3500 3000 2500 2000 1500 1000 500 0 -500
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
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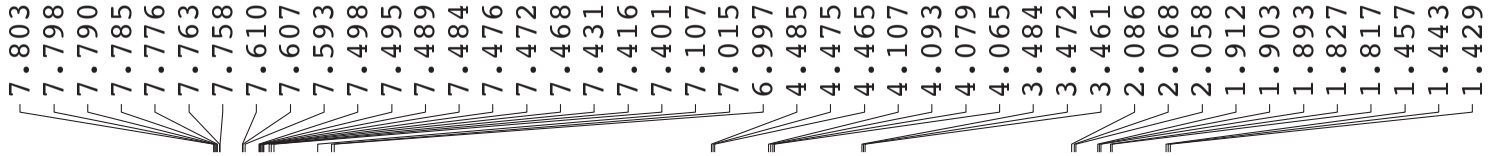

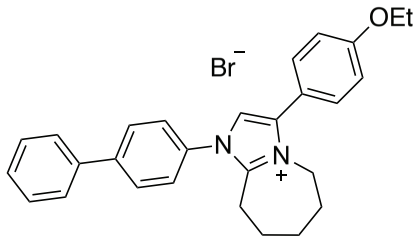

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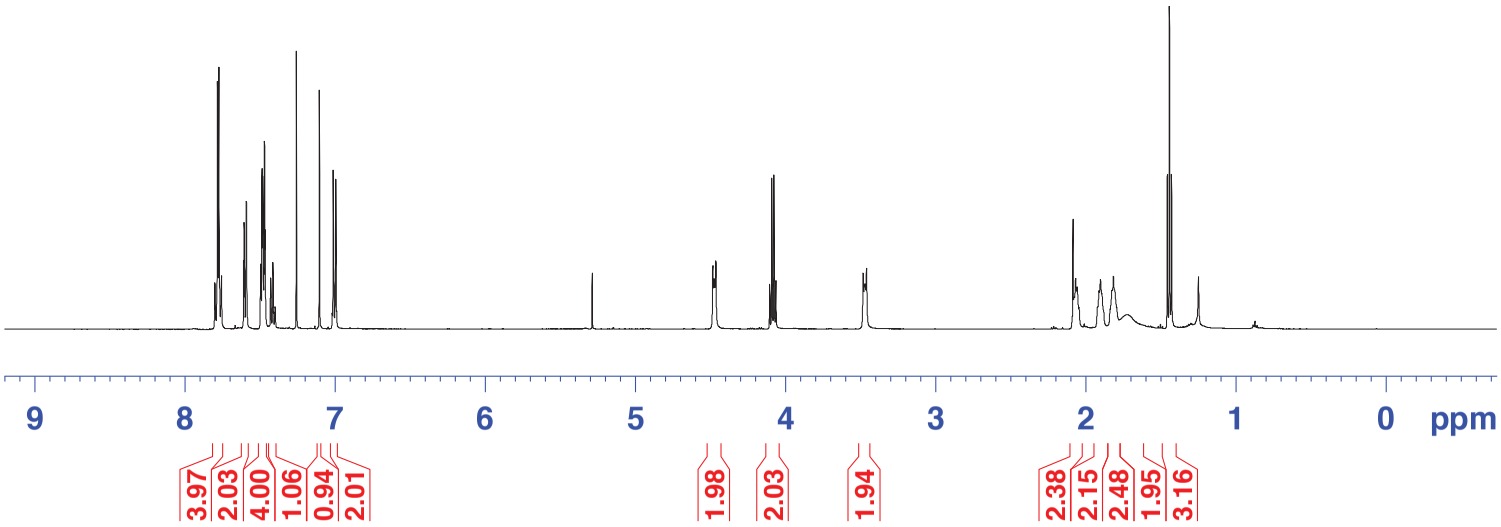

Current Data Parameters

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PROCNO	1

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FIDRES	0.157632 Hz
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RG	203
DW	48.400 usec
DE	6.50 usec
TE	303.2 K
D1	1.00000000 sec

======== CHANNEL f1 ========

NUC1	1H
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PLW1	20.00000000 W
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F2 - Processing parameters

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SSB	0
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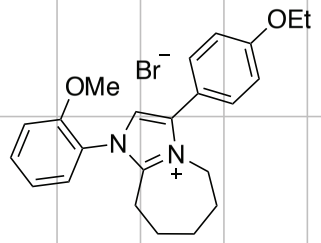
STANDARD PHOSPHORUS PARAMETERS



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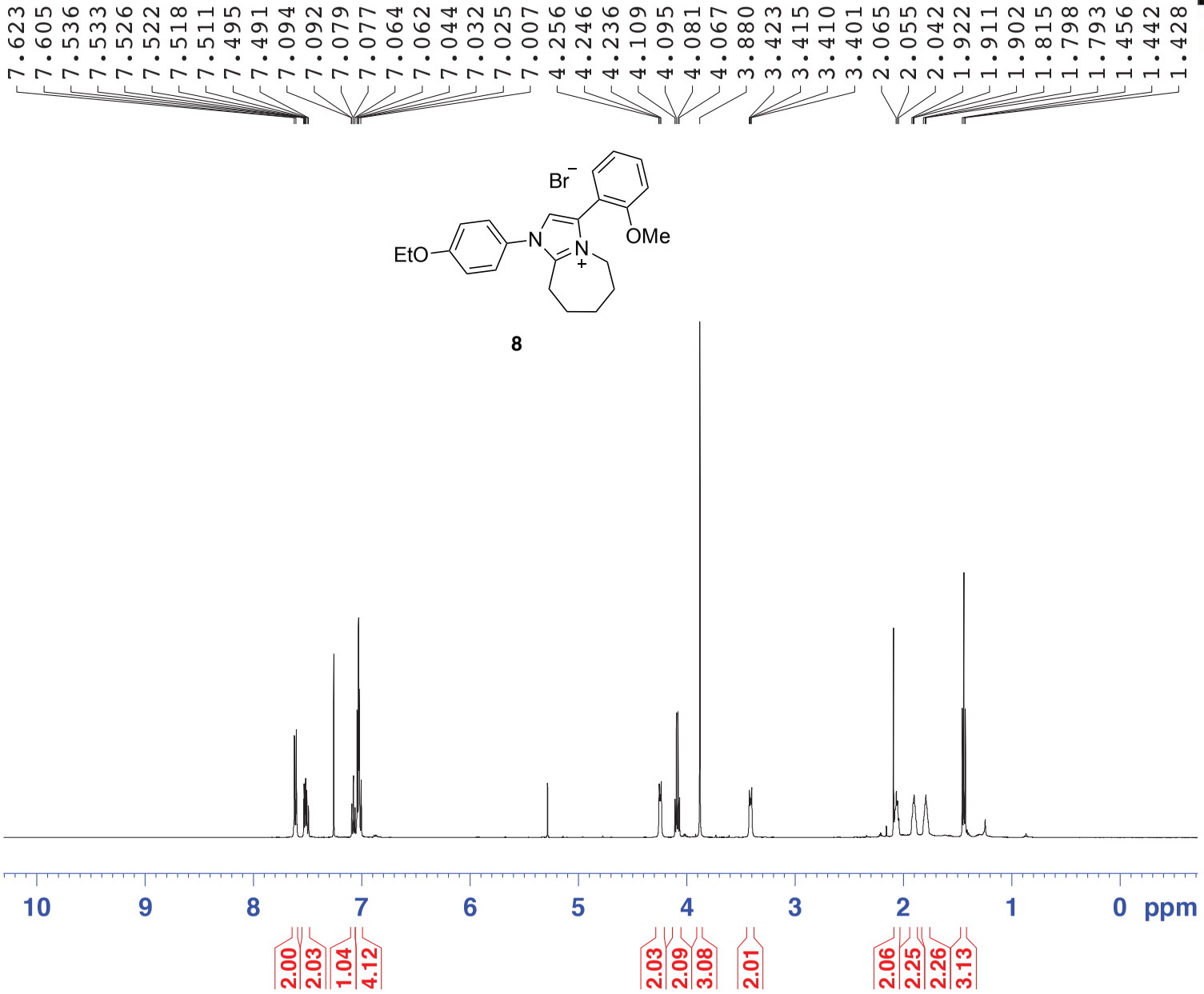
f1 (ppm)



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PROCNO	1

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DW	48.400 usec
DE	6.50 usec
TE	303.2 K
D1	1.00000000 sec

======== CHANNEL f1 ========

NUC1	1H
P1	10.13 usec
PLW1	20.00000000 W
SFO1	500.1330885 MHz

F2 - Processing parameters

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SF	500.1300141 MHz
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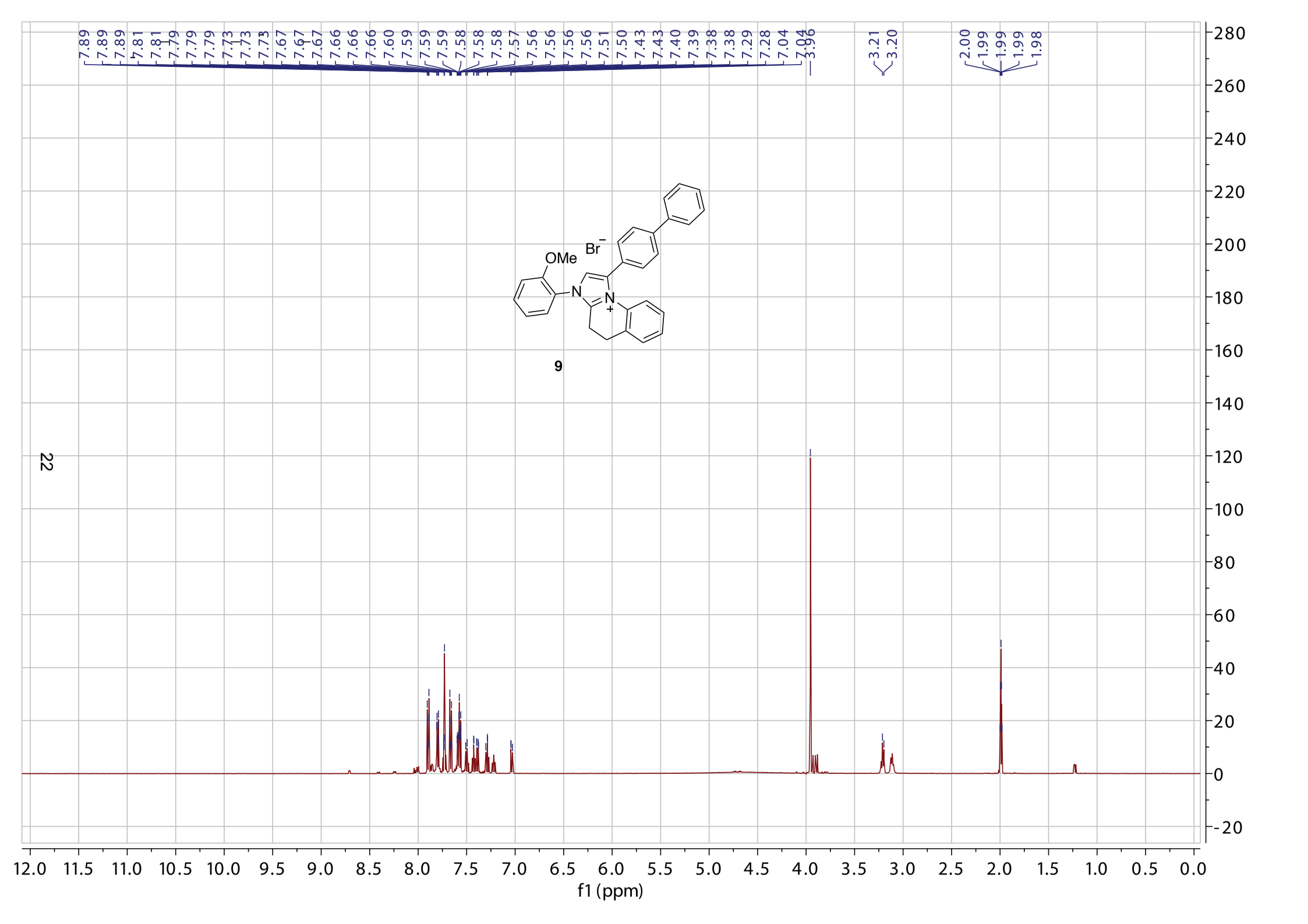
PC	1.00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3.20
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OMe
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120
100
80
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11.0
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7.5
7.0
6.5
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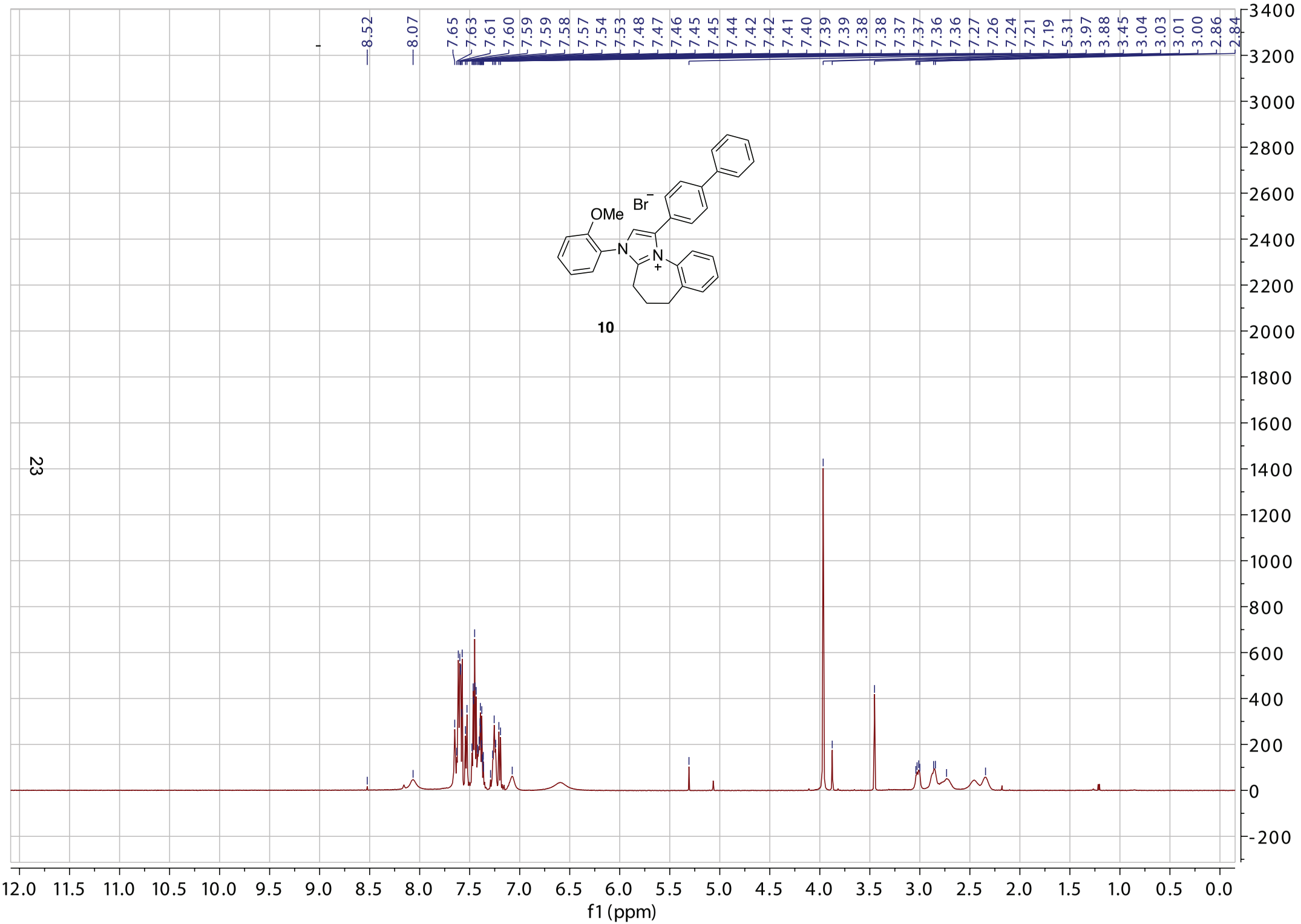
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7.21
7.19
5.31
3.97
3.88
3.45
3.04
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3.01
3.00
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OMe
Br
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N
+
10
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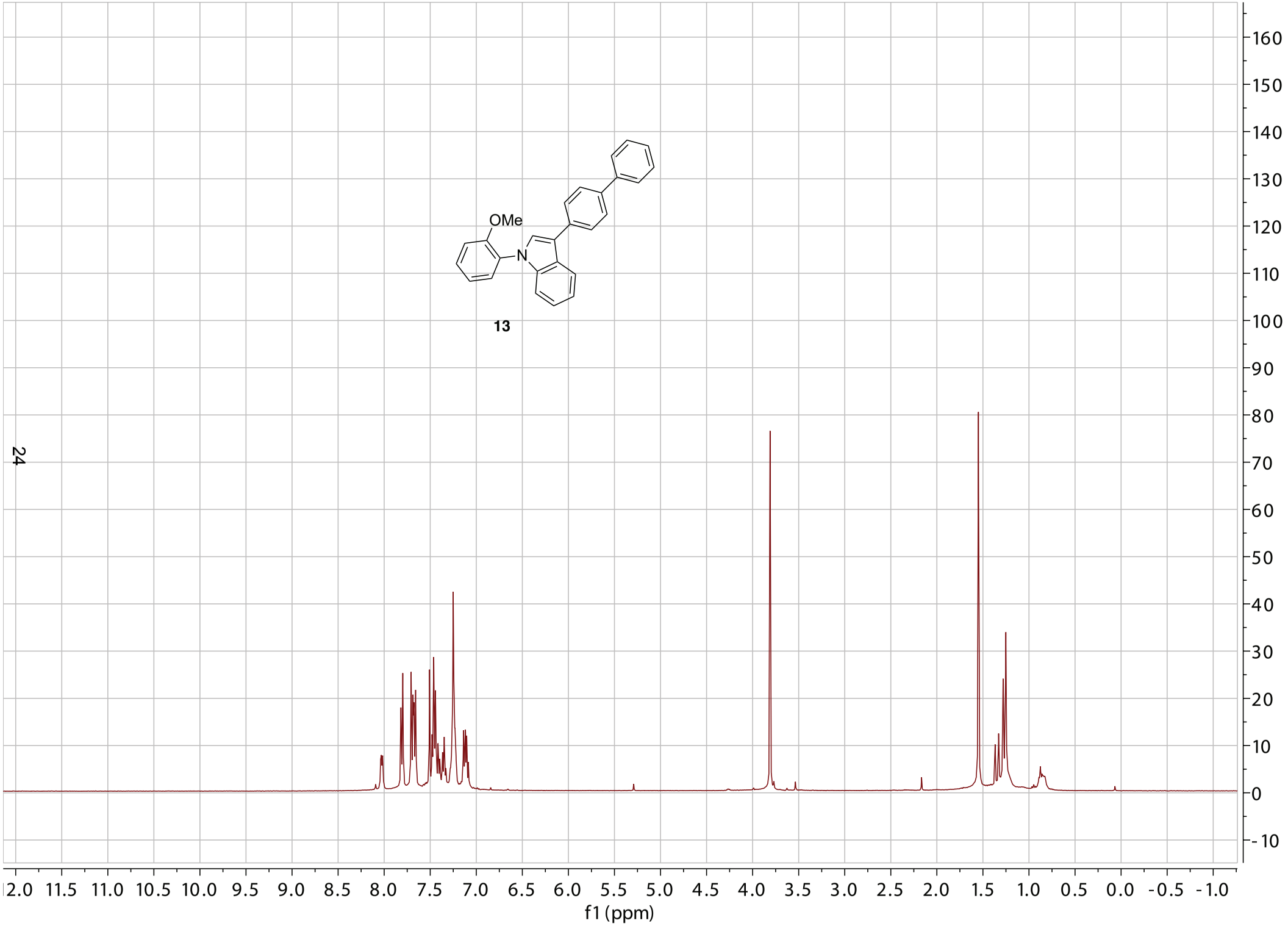
f1 (ppm)


OMe
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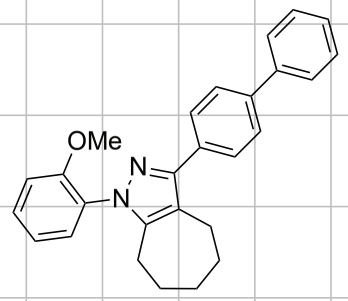
f1 (ppm)



14

25

f1 (ppm)

Materials and Methods

High-throughput screen for small-molecule antagonists of Gli function

The high-throughput chemical screen for inhibitor of Gli function was contracted to the Conrad Prebys Center for Chemical Genomics at the Sanford Burnham Prebys Medical Discovery Institute. The screen conditions were equivalent to those described in PubChem AID 588413. In brief, 5 x 10^3 SUFU-KO-LIGHT cells were seeded into white 1536-well plates. 16 to 18 hours after seeding, the cells were treated with individual compounds at the final concentration of 5 μM and cultured for another 16 to 18 hours. The cells were then treated with Bright-Glo luciferase substrate (3 μL/well; Promega) for 10 minutes at room temperature before luminescence was read on a Viewlux microplate imager. Hits with at least 50% luciferase inhibition were expanded for a dose-response assay, and those with IC50s lower than 20 μM were subsequently counter-screened in cytotoxicity, luciferase, and WNT assays.

Cell lines and cell culture

SUFU-KO-LIGHT: DMEM containing 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% penicillin/streptomycin (pen/strep), 150 μg/mL zeocin, and 400 μg/mL geneticin

NIH-3T3 and NIH-3T3 FLAG-GLI1: DMEM containing 10% calf serum (CS), 1% sodium pyruvate, and 1% pen/strep

NIH-3T3-ρ^0: DMEM containing 10% CS, 1% sodium pyruvate, 1% pen/strep , 100 ng/mL ethidium bromide, and 50 μg/mL uridine (minimum of 6 days to ensure loss of functioning electron transport chain)

C3H10T1/2: DMEM containing 10% FBS, 1% sodium pyruvate, and 1% pen/strep

Murine embryonic fibroblasts (WT and *Gli1*$^{-/-}$[1] MEFs): DMEM containing 10% CS, 1% sodium pyruvate, 1% pen/strep, and 1% non-essential amino acids

Wnt3a-expressing L: DMEM containing 10% FBS, 1% pen/strep

WNT-LIGHT: DMEM containing 10% FBS, 1% pen/strep , and 1.5 mM geneticin

SHH-producing HEK 293: DMEM containing 10% FBS, 1% sodium pyruvate, 1% pen/strep, 1.5 mM geneticin

All cell lines were grown at 37 °C with 5% CO_2. Serum starvation was performed in media lacking phenol red and containing either 0.5% FBS or CS.

Generation of stable cell lines

SUFU-KO-LIGHT: Embryonic fibroblasts derived from *Sufu* knockout mice (*Sufu*$^{-/-}$ MEFs[2]) were co-transfected with a zeocin resistance vector and a firefly luciferase reporter driven by eight tandem Gli-binding sites and a basal δ-crystallin promoter (8xGliBS:luciferase). After selection, the cell line was co-transfected with SV40 promoter-driven *Renilla* luciferase reporter (pRLSV40; Promega) and a puromycin resistance vector. Dilution cloning in puromycin-containing medium (1 μg/mL) then yielded the SUFU-KO-LIGHT line.

WNT-LIGHT: L-cells (ATCC) were co-transfected with the SuperTopFlash reporter[3], which contains seven tandem TCF/LCF binding sites and the firefly luciferase coding region, pRLSV40, and pcDNA3 (Invitrogen; for geneticin resistance). Ring cloning in geneticin-containing medium (1 mg/mL) then yielded the WNT-LIGHT line.

NIH-3T3 FLAG-GLI1: NIH-3T3 cells were retrovirally transduced with pBMN-TAG2-IRES-mCherry containing the mouse *Gli1* coding sequence at a multiplicity of infection (MOI) of 0.3. Two days after infection, mCherry-positive cells were isolated by fluorescence-activated cell sorting and expanded.

SHH-conditioned medium
The SHH N-terminal domain was stably expressed in HEK-293T-EcR cells. Cells were grown to 80% confluency, and medium was changed to DMEM containing 2% FBS. SHH-conditioned medium was collected after two days and sterilized using a 0.22-µm filter. The SHH titer was determined by adding serial dilutions of conditioned medium to SHH-LIGHT2 cells, an NIH-3T3 fibroblast-derived line that is stably transfected with 8xGliBS:luciferase and pRLSV40 reporters. SHH-conditioned medium was used at a concentration two-fold greater than minimum dilution required for maximum SHH pathway activation (typically 1:10).

WNT3A-conditioned medium
WNT3A ligand was stably expressed in L cells. After the cells reached 100% confluency, the medium was collected, centrifuged at 500 *g* for 10 minutes, passed through a 0.22-µm filter, and stored for up to two weeks at 4 °C.

Western blot
Samples were lysed in 1X SDS-PAGE loading buffer, boiled, sonicated, and resolved on a 3-8% Tris-acetate Criterion gel (Bio-Rad) in XT Tricine buffer. Proteins were then transferred to a PVDF membrane using a Bio-Rad Transblot Turbo system. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) or 1:1 phosphate-buffered saline (PBS)/Seablock (for GLI1) for 1 hour at room temperature. The blots were then incubated with primary antibody overnight at 4 °C and with HRP-conjugated secondary antibody for 1 hour at room temperature. Chemiluminescent detection of the HRP-conjugated secondary antibodies was conducted with Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and a ChemiDoc MP imaging system (Bio-Rad). Image quantification was performed using ImageJ.

qPCR
NIH-3T3 cells and *Gli1*$^{-/-}$ MEFs were seeded into 6-well plates at a density of 6.5 x 10^5 cells/well or 24-well plates at a density of 1.2 x 10^5 cells/well. After 24 hours, the cells were cultured in serum starvation medium containing various concentrations of compound (with or without SHH) for 24 hours, unless indicated otherwise. After 24 hours, RNA was extracted using the Monarch Total RNA Miniprep kit, and the SuperScript III First-Strand Synthesis kit was used to generate cDNA. Samples were incubated with TaqMan FAM probes, and qPCR was performed on a Roche LightCycler 480. Cycle numbers were normalized to the housekeeping gene B2M.

Immunofluorescence microscopy
NIH-3T3 cells were seeded onto poly-D-Lysine-coated coverslips in 24-well plates at a density of 1.2 x 10^5 cells/well. After 24 hours, the cells were cultured in serum starvation medium containing various concentrations of compound (with or without SHH) for the indicated times.

To determine the effects of compounds on ciliary GLI2 levels, NIH-3T3 cells were pre-treated with SHH for 24 hours and then cultured with the indicated compounds for 6 hours. The cells were subsquently fixed in 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with ice-cold methanol for 5 minutes at -20 °C. Coverslips were stained with primary antibodies (GLI2, ARL13B, and γ-tubulin), Alexa Fluor dye-labeled secondary antibodies, and DAPI and then imaged with a Zeiss Axio Imager M1 eplfluorescence microscope controlled by SlideBook software (Intelligent Imaging Innovations). GLI2 ciliary tip intensity was determined as previously described[4]. Briefly, a ciliary mask was generated using ARL13B immunofluorescence, and γ-tubulin staining was used to identify the cilium base. Ciliary GLI2 levels were determined by integrating GLI2 immunofluorescence signals in the last five pixels of the distal tip.

To evaluate the subcellular localization of FLAG-GLI1, NIH-3T3 cells were treated with the indicated compounds for 4 hours, fixed with 4% paraformaldehyde for 10 minutes at room temperature, and permeabilized with 0.3% Triton X-100. Coverslips were stained with anti-FLAG primary antibody overnight at 4 °C , and the following day with Alex Fluor 488 IgG secondary antibody for 1 hour at room temperature in the dark, and mounted with DAPI. The immunostained cells were then imaged on a Zeiss LSM 800 confocal microscope equipped with ZEN Blue software. The ratio of cytoplasmic:nuclear FLAG-GLI1 was

determined by creating a mask based on the DAPI signal and comparing the average pixel intensities within the mask (nuclear) and at least 10 pixels outside of the mask (cytoplasmic).

To assess mitochondrial morphology, NIH-3T3 cells co-treated with SHH and the indicated compounds for 24 hours and then stained with 500 nM MitoTracker Deep Red FM for 30 minutes at 37 °C. Cells were fixed with 4% paraformaldehyde and permeabilized in ice-cold methanol for 15 minutes at -20 °C. Coverslips were imaged on a Zeiss LSM 800 with ZEN Blue software. Mitochondrial morphology was manually scored for normal (elongated and branched) and disrupted (perinuclear and rounded) phenotypes.

SUFU-KO-LIGHT assay
SUFU-KO-LIGHT cells were seeded into 96-well plates at a density of 3.5 x 10^4 cells/well. After 24 hours, the cells were cultured in serum starvation medium containing the indicated compounds for another 24 hours. 20 µL/well CellTiter AQueous One (Promega) was then added to the cells and incubated for 30 minutes at 37 °C. Cell viability was measured as absorbance at 490 nm of the culture media on a Molecular Devices Spectramax M2e. Cells were then washed with PBS and incubated with 100 µL/well Bright-Glo reagent for 5 minutes at room temperature. Gli-dependent firefly luciferase activity was measured on a Veritas luminometer and normalized to the CellTiter signal. Dose-response curves were generated using Prism software (GraphPad).

WNT-LIGHT assay
WNT-LIGHT cells were seeded into 96-well plates at a density of 1 x 10^4 cells/well. After 24 hours, the cells were cultured in a 1:1 ratio of WNT3A-conditioned medium and serum starvation medium for another 24 hours. The cells were then lysed using a Dual Luciferase kit (Promega), and TCF/LEF-dependent firefly luciferase activity was measured on a Veritas luminometer and normalized to *Renilla* luciferase levels.

CMV-luciferase assay
NIH-3T3 cells were seeded into a 24-well plate at density of 3.5 x 10^4 cells/well. After 24 hours, the cells were approximately 50% confluent and co-transfected with pRLSV40 (5 ng/well), a CMV-driven firefly luciferase expression vector (15 ng/well), and TransIT-LT1 reagent (Mirus Bio). The medium was changed the following day, and the cells were cultured for an additional 24 hours until they reached 100% confluency. The cells were then cultured in serum starvation medium containing the indicated compounds for another 24 hours. Cells were then lysed using the Dual Luciferase kit, and firefly and *Renilla* luciferase activities were measured on a Veritas luminometer.

Alkaline phosphatase assay
C3H10T1/2 cells were seeded into a 96-well plate at a density of 2.5 x 10^4 cells/well. After 24 hours, the cells were cultured for another 48 hours in a serum starvation medium containing 10% SHH-conditioned medium and the indicated compounds. The cells were then treated with 50 µL lysis buffer (100 mM Tris-HCl, pH 9.5, 250 mM NaCl, 25 mM $MgCl_2$, and 1% Triton X-100) and rocked for 45 minutes at room temperature. 10 µL of lysate was transferred to a white-wall 96-well plate and incubated with 50 µL CDP-Star reagent (NEB) for 15 minutes at room temperature in the dark. Luminescence was measured on a Veritas luminometer.

Seahorse XF Mito Stress assay
NIH-3T3 or NIH-3T3-ρ^0 cells were seeded into an XF96 plate at a density of 9 x 10^3 cells/well. After 24 hours, the cells were incubated for 1 hour at 37 °C in a non-CO_2 incubator. Cells were then loaded into a 96-well Seahorse XF analyzer (Agilent) and sequentially injected with the indicated compounds, 1 µM oligomycin A, 1 µM FCCP, or 0.5 µM rotenone/antimycin according to the Agilent Mito Stress protocol. The oxygen consumption rate (OCR, pmol/min) was measured every 5 minutes for a 15-minute period, and oxidative phosphorylation capacity was determined by background-subtracting the average oligomycin value from of each reading, and normalizing the data to the DMSO control.

NCI-60 cancer cell line proliferation and COMPARE analysis
Inhibitor **5** was tested by the NIH against its panel of approximately 60 human cancer cell lines at 5 different doses (100 µM, 10 µM, 1 µM, 0.1 µM, and 0.01 µM) over the course of 7 days according the NCI-60

screening methodology[5]. COMPARE analysis was then performed by cross-correlating the NCI-60 inhibition profile for inhibitor **5** against those of over 80,000 synthetic compounds.

Nuclear and cytoplasmic fractionation

FLAG-GLI1-expressing NIH-3T3 cells were seeded into 6-well plates at a density of 6 x 10^5 cells/well. After 24 hours, cells were treated with indicated compounds for 24 hours. The cells were then lysed and separated into nuclear and cytoplasmic fractions using an NE-PER kit (Thermo Fisher Scientific), according to the manufacturer's protocols.

Mitochondrial membrane potential

NIH-3T3 cells were seeded into 24-well plates at a density of 1.2 x 10^5 cells/well. After 24 hours, the cells were cultured for another 24 hours in serum starvation medium containing 10% SHH-conditioned medium and the indicated compounds. The medium was then supplemented with TMRM and Mitotracker Deep Red (500 nM final concentrations for each) and DAPI, and the cells were incubated in this medium for 30 minutes at 37 °C. Cells were then washed, maintained in serum starvation medium without phenol red, and imaged on a BZ-X700 Keyence microscope equipped with BZ-X analyzer software.

References

[1] R. J. Lipinski, M. F. Bijlsma, J. J. Gipp, D. J. Podhaizer, W. Bushman, *BMC Cell Biol.* **2008**, *9*, 1–8.
[2] M. H. Chen, C. W. Wilson, Y. J. Li, K. K. Lo Law, C. S. Lu, R. Gacayan, X. Zhang, C. C. Hui, P. T. Chuang, *Genes Dev.* **2009**, *23*, 1910–1928.
[3] M. T. Veeman, D. C. Slusarski, A. Kaykas, S. Hallagain Louie, R. Moon, *Curr. Biol.* **2003**, *13*, 680–685.
[4] D. K. Breslow, S. Hoogendoorn, A. R. Kopp, D. W. Morgens, B. K. Vu, M. C. Kennedy, K. Han, A. Li, G. T. Hess, M. C. Bassik, et al., *Nat. Genet.* **2018**, *50*, 460–471.
[5] R. H. Shoemaker, *Nat. Rev. Cancer* **2006**, *6*, 813–823.

	Reagent	Supplier and Catalog Number
Primary antibodies		
	GLI1	Cell Signaling Technologies L42B10
	GLI2	R&D systems AF3635
	GLI3	R&D Systems AF3690
	FLAG	Sigam-Aldrich F1804
	Karyopherin subunit beta 1	Santa Cruz sc-137016
	Arl13b	UC Davis/NIH NeuroMab Clone N295B/66
	Gamma-tubulin	Sigma Aldrich T6557
Secondary antibodies		
	Affinipure goat anti-mouse IgG (H+L)	Jackson Immunoresearch #115-005-166
	Bovine anti-goat IgG (H+L)	Jackson Immunoresearch #805-035-180
	Sheep anti-mouse IgG	GE Sciences NA931
TaqMan probes		
	Gli1	Mm00494645
	Ptch1	Mm00436026
	B2M	Mm00437762
Kits		
	Seahorse XF Mito Stress Test	Agilent Technologies 103015
	Seahorse XFe FluxPak	Agilent Technologies 102601
	NE-PER nuclear and cytoplasmic kit	Thermo Fisher 78833
	Dual-Luciferase reporter system	Promega E1910
	Bright-Glo luciferase system	Promega E2610
Compounds/Other		
	Cyclopamine	Sigma Aldrich C4116
	Oligomycin A	Sigma Aldrich 75351
	Tetramethylrhodamine	Thermo Fisher T668
	CDP-Star substrate (0.25 mM)	Thermo Fisher T2146
	L Wnt-3A cells	ATCC 2647
	DAPI ProLong Gold Antifade	Thermo Fisher P36931
	Mitotracker Deep Red	M22426
Bicyclic imidazolium analogs	Bicyclic imidazolium **1**	MolPort, MolPort-007-793-026
	Bicyclic imidazolium **4**	Princeton BioMolecular Research, OSSK_001861
	Bicyclic imidazolium **11**	Aurora Fine Chemicals LLC, K00.358.921
	Bicyclic imidazolium **12**	Aurora Fine Chemicals LLC, A35.152.540